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RESEARCH PAPER

Development of a rapid method for the analysis of trenbolone, nortestosterone, and zeranol in bovine liver using liquid chromatography tandem mass spectrometry

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Abstract A rapid liquid chromatography tandem mass spectrometry method has been developed and validated for the determination of α-trenbolone, β-trenbolone, α-nortestosterone, β-nortestosterone, zeranol, and taleranol in bovine liver. The impact of liquid–liquid extraction with methyl tert-butyl ether and optimized solid phase extraction on silica cartridges significantly reduced effort and time of sample preparation. Electrospray ionization gives a significant signal increase compared with atmospheric pressure chemical ionization and atmospheric pressure photoionization. The HPLC gradient was optimized to separate isobaric analytes and matrix constituents from the hormone molecules. The optimized time and temperature of enzymatic hydrolysis of conjugated trenbolone was 4 h at 52 °C. The method validated in the range of 0.5–30 μg kg^{-1} for α-trenbolone, β-trenbolone, zeranol, taleranol, and 2–30 μg kg^{-1} for α-nortestosterone, β-nortestosterone. Combined uncertainty of measurements was in the range of 4 %–23 %. The matrix effect was negligible (1 %–5 %) for all analytes except of α-nortestosterone (19 %). The developed method with changes concerning sample size and hydrolysis was also applied for the analysis of meat, serum, and urine samples.

Keywords LC-MS/MS · Bovine liver · Trenbolone · Nortestosterone · Zeranol

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Introduction

Trenbolone, nortestosterone, and zeranol are hormonally active substances that are used for growth promotion, resulting in an improvement of muscle growth, lean meat, and higher feed conversion efficiency for food producing animals [1, 2]. However, application of them to food producing animals poses a risk for public health because of their potential endocrine disrupting properties. As a consequence, the use of anabolic agents for growth promotion purposes has been banned in the European Union [3] and Russia [4]. Therefore, the monitoring of these residues is necessary to ensure that there was no abuse of hormonally active substances.

Trenbolone and nortestosterone are anabolic steroids with androgenic activity, derivatives of testosterone used to improve feed conversion rate and carcass characteristics of farm livestock. Zeranol and its primary metabolite taleranol are resorcylic acid lactones. Zeranol has been widely used as a growth stimulant with estrogenic activity [5, 6]. The chemical structures of these analytes are shown in Figure 1.

From the point of view of biodistribution, liver is one of the most suitable matrices for the control of growth promoters abuse in cattle, along with urine, kidney, bile, and plasma [7]. However, when monitoring for misuse of these compounds from countries exporting beef, usually only liver and muscle are available for analysis. Over the years, various analytical procedures such as liquid–liquid extraction (LLE), solid phase microextraction, solid–liquid extraction, and solid phase extraction (SPE) have been developed in order to achieve the aim of monitoring for the use of illegal growth promoters in meat samples. In the protocols reported in the literature, combinations of the above-mentioned analytical procedures are used for the determination of anabolic steroids with satisfactory results [8, 9]. But the analysis of liver is a more challenging task compared with meat analysis because of more complicated sample preparation. The Commission of

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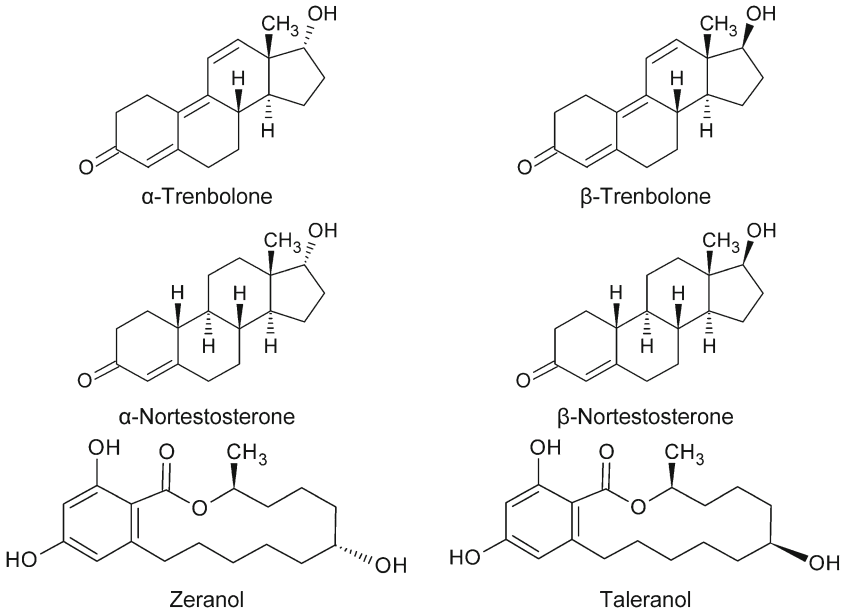

Fig. 1 Chemical structures of target analytes

the European Communities has presented a routine sample preparation procedure for analysis of liver samples using column chromatography [10]. In other procedures, immunoaffinity chromatography [11] and multi-step solid phase extraction [12–15] have been used. All these procedures are time-consuming and require large volumes of solvents. An overview of the main characteristics of the sample preparation for these methods is given in Table 1.

This paper describes a simple, rapid, and reliable LC-MS/MS method that involves one-step solid phase extraction for the determination of α-trenbolone, β-trenbolone, α-nortestosterone, β-nortestosterone, zeranol, and taleranol in bovine liver.

Experimental

Chemicals and reagents

17α-Nortestosterone, 17β-nortestosterone, 17β-nortestosterone-D3, 17α-trenbolone, 17β-trenbolone, 17β-trenbolone-D3, zeranol, taleranol, and taleranol-D4 were obtained from RIKILT (Wageningen, The Netherlands). Primary stock solutions were prepared in methanol at a concentration of 100 μg mL^{-1}. Working solutions were prepared in methanol/water (50/50) at a concentration of 1 μg mL^{-1}. Methanol, methyl tert-butyl ether (MTBE), n-hexane, β-glucuronidase/arylsulfatase from *Helix pomatia* were obtained from Merck (Darmstadt, Germany), acetic acid, formic acid were from Sigma (Steinhem, Germany), sodium acetate was from ROTH (Karlsruhe, Germany), and toluene and acetone were from Acros Organics (Fair Lawn, NJ, USA). All solvents were of HPLC grade.

Sodium acetate buffer 0.2 M was prepared by dissolving 16.4 g sodium acetate in 900 mL of water and adjusting pH to 5.2 using acetic acid. Water was added to reach a final volume of 1000 mL. Ultrapure water was produced with Millipore Direct-Q 5 system (Molsheim, France).

The silica SPE cartridges were prepared as follows: 20 μm polypropylene frit (Agilent, part no. 12131022, Santa Clara, CA, USA) inserted in 12 mLSPE cartridge (Agilent Bond Elute, part no. 12131010, Santa Clara, CA, USA), then cartridge packed with 0.5 g of silica gel sorbent (Merck, part no. 115101, Darmstadt, Germany) and another frit inserted to fix the sorbent in the cartridge.

Sample preparation

Liver samples (5.0 g) were weighed into 50 mL centrifuge tubes. Samples were fortified with mixed internal standard at a level corresponding to 10 μg kg^{-1} by adding 50 μL of 1000 ng mL^{-1} internal standard mix solution. After fortification, samples were held for 15 min, then 6 mL of sodium acetate buffer was added and the samples were homogenized with a WiseTis homogenizer (Daihan, Seoul, Republic of Korea) for about 1 min. The pH of each mixture was readjusted to 5.2 and 50 μL glucuronidase/arylsulfatase from *Helix pomatia* was added and incubated at 52 °C for 4 h. After cooling down to room temperature, the mixture was extracted with 15 ml MTBE (10 min rotating and centrifuged at 3000 rpm). The organic layer was evaporated to dryness and the residue was redissolved in 3.0 mL of hexane/acetone (90/10, v/v). Silica gel SPE cartridges were preconditioned with 8 mL of hexane/acetone (90/10, v/v) and the samples were applied to the cartridges. After washing the cartridges with 10 mL of hexane/acetone (90/10, v/v) the analytes were eluted

Table 1 Overview of the main characteristics of the different sample preparation methods for the analysis of bovine liver samples

Reference	Sample weight	Hydrolysis	Defatting	Clean-up	Method	LOQ, μg kg^{-1}
[10]	50 g	Helix pomatia juice 37 °C, one night	Hexane	Clean-up using Amberlite XAD 2, Celite, and Al_2O_3 columns	GC-MS	1
[11]	5 g	Helix pomatia juice 37 °C, 2 h	Petroleum ether	Clean-up using immunoaffinity chromatography or HPLC columns	GC-MS	0.5-2
[12]	5 g	Without hydrolysis. The analytes were extracted with 100 mL of 0.2 % metaphosphoric acid–acetonitrile (6:4, v/v) following by evaporation under reduced pressure	-	SPE on Waters Oasis HLB cartridges (the volume of sample passed through the cartridge – 30 mL)	HPLC-ESI-MS	0.5
[13]	10 g	Helix pomatia juice 54 °C, one night	Hexane	1. SPE on Waters Oasis HLB cartridge (the volume of sample passed through the cartridge – 100 mL) 2. SPE on Waters Sep-pak Silica cartridge 3. SPE on Waters Sep-Pak Amino Propyl cartridge	HPLC-ESI-MS/MS	0.05–0.3
[14]	5 g	Helix pomatia juice 37 °C, one night	-	1. SPE on Supelco GCB cartridge (the volume of sample passed through the cartridge – 125 mL) 2. SPE on Waters Sep-Pak Amino Propyl cartridge	UHPLC-ESI-MS/MS	Data not provided
[15]	10 g	Without hydrolysis. The analytes were extracted with 45 mL of 0.2 M acetate buffer/ methanol mixture	-	1. SPE on Waters Oasis HLB cartridge (the volume of sample passed through the cartridge – 100 mL) 2. SPE on Waters Sep-pak Silica cartridge 3. SPE on Waters Sep-Pak Amino Propyl cartridge	HPLC-ESI-MS	1

using 10 mL of hexane/acetone (80/20, v/v). The eluents were evaporated to dryness, redissolved in 1000 μL of methanol/water (80/20, v/v) and centrifuged 10 min at 5200*g*, 4 °C. The supernatant was transferred to a vial and analyzed by LC-MS/MS.

LC conditions

A binary solvent delivery system (Eksigent UltraLC-100; Eksigent, Dublin, CA, USA), including a binary pump and a degasser, was used. Chromatographic separation was achieved by reversed phase chromatography and gradient elution. Separation of the analytes was carried out on a Pursuit 3 C18 column (150 mm×2.1 mm, particle size 5 μm, Agilent), maintained at 40 °C. The autosampler temperature was set to 4 °C and the injection volume was 20 μL. Samples were injected twice: once in positive polarity mode and once in negative one. The mobile phases were water (phase A) and methanol (phase B). In positive mode, a linear gradient was applied at a flow rate of 200 $\mu L min^{-1}$ starting at 100 % A, increasing to 80 % B within 25 min, and keeping 80 % B for 5 min. Subsequently, the column was re-equilibrated for 10 min at 100 % A. In negative mode, a linear gradient used was as follows: 40 % B at the start, increased linear to 60 % within 4 min, held for 9 min, and equilibrated for 10 min at the initial conditions. The flow rate was kept at 150 $\mu L min^{-1}$.

MS/MS parameters

A triple quadrupole mass spectrometer (QTRAP 5500; AB SCIEX, Toronto, ON, Canada) was used with electrospray ionization, atmospheric pressure chemical ionization, and atmospheric pressure photoionization sources. At least two transitions per analyte were monitored, whereas only one transition was monitored for each deuterated internal standard. The transitions for each analyte as well as the corresponding collision energies are shown in Table 2. Declustering potential and collision cell exit potential were 100 and 14 V in positive mode, and –110 and –20 V in negative mode, respectively, for all analytes. The resolution of quadrupole 1 (Q1) and quadrupole 3 (Q3) was set to "unit." Parameters of ESI, APCI, and APPI sources are shown in Table 3. Eksigent UltraLC-100 pump was used for dopant (toluene) delivery in APPI at flow rate 30 μL/min. The data were acquired and processed using Analyst software ver. 1.6.1 (AB SCIEX). Identification and confirmation of analytes were performed in accordance with EC/2002/657 [16].

Matrix effect

Matrix effect (M) was studied using ESI mode and was evaluated by calculating the percentage of signal suppression according to the following formula [17]: $M\ (\%)=(1-S_m/S_s)\times 100$, where S_m is the slope of calibration curve prepared by fortifying blank liver samples after sample preparation at four concentration levels (0.5, 2, 8, and 30 $\mu g\ kg^{-1}$) and S_s is the slope of calibration curve constructed in pure solvent at the same concentrations.

In-house validation

The validation experiment was based on full factorial design for two factors and consisted of four runs. A run contains blank liver samples fortified at 0.5, 2, 8, and 30 $\mu g\ kg^{-1}$, which were analyzed in two replicates and matrix-matched calibration samples fortified at the same levels. Operator and storage of extract after sample preparation were selected as factors that cannot be controlled in routine analysis. In order to prove the specificity and the lack of susceptibility to matrix interferences, several blank samples fortified with internal standard were additionally analyzed in each runs.

Samples

Meat and liver samples were bought at the local supermarket. Serum and urine samples were collected from animals of both sexes at a farm in Moscow. All samples were analyzed prior to use and were determined to be free of the analytes.

Table 2 MS/MS parameters and retention time of investigated analytes and their internal standards

Compound	Retention time (min)	Parent ion (*m/z*)	Daughter ion (*m/z*)	Collision energy (eV)	Polarity
α-Trenbolone	23.1	271.0	199.1[a], 165.1, 253.1	27, 71, 27	Positive
β-Trenbolone	22.5	271.0	199.1[a], 165.1, 253.1	27, 71, 27	Positive
β-Trenbolone-D3	22.5	274.1	199.1	27	Positive
α-Nortestosterone	24.5	275.2	109.2 [a], 257.2	35, 24	Positive
β-Nortestosterone	23.3	275.2	109.2 [a], 257.2	35, 24	Positive
β-Nortestosterone-D3	23.3	278.2	242.1	24	Positive
Zeranol	8.9	321.2	277.2 [a], 303.2	-30, -30	Negative
Taleranol	7.7	321.2	277.2 [a], 303.2	-30, -30	Negative
Taleranol-D4	7.7	325.1	281.1	-35	Negative

[a] The ion used for quantitative analysis

Table 3 Parameters of ESI, APCI and APPI sources

Parameters	ESI		APCI		APPI	
	Positive	Negative	Positive	Negative	Positive	Negative
Turbogas (Gas 2)	40	30	-	-	-	-
Nebulizer gas	60	50	40	40	40	40
Lamp gas	-	-	-	-	20	20
Curtain gas	15	15	20	20	20	20
Collision gas	Medium	Medium	Medium	Medium	Medium	Medium
Ion spray voltage, V	5500	–4500	-	-	800	–750
Turbogas temperature, °C	500	450	-	-	-	-
Probe temperature, °C	-	-	400	400	400	400
Needle current, μA	-	-	2	-2	-	-
Lamp vertical position, mm	-	-	-	-	4	4

Results and discussion

Optimization of sample preparation

To simplify sample preparation, we proposed the use of liquid–liquid extraction with MTBE, which was previously used in the analysis of anabolic agents in the urine, serum, and meat of cattle [8, 18–20]. The residue obtained after evaporation of MTBE contained about 0.5 mL of fat, so the next task was the optimization of defatting step. The use of LLE with hexane was abandoned because of the complexity of the process and the low degree of purification of the extract. Instead of LLE with hexane, SPE on silica gel cartridges was proposed. Different ratios of hexane and acetone for cartridge washing and elution of analytes were investigated on bovine liver samples fortified at 2 μg kg^{-1}. Washing with hexane (without acetone) resulted in a dirty extract after reconstitution, whereas adding more than 15 % of acetone resulted in eluting of analytes from cartridge. The optimal washing hexane/acetone ratio was 90/10 (v/v).

After SPE optimization conditions for enzymatic hydrolysis of α-trenbolone conjugates were investigated. Enzymatic hydrolysis is necessary since studies conducted on heifers implanted with trenbolone acetate according to label instructions indicated that residues of both α-trenbolone and β-trenbolone were present, primarily as conjugates in liver, with α-trenbolone conjugates being at approximately four times the concentration of β-trenbolone conjugates [21]. The incubation time and temperature were optimized by the analysis of liver sample that contained incurred residues of trenbolone for four time and temperature combinations. The results showed that incubation during 4 h at 52 °C gives the maximum signal intensity of α-trenbolone, whereas the incubation during one night decrease signal intensity in 20 % (Figure 2). The final optimized scheme of sample preparation is shown in Figure 3.

LC-MS/MS analysis

In the majority of the published methods for analysis of anabolic agents in urine, serum, and meat samples, ESI is applied [8]. But in some works [8, 19, 20, 22, 23] APCI was used because of higher detection sensitivities for most steroidal compounds compared with ESI. Various results obtained by different authors using the same ionization techniques may be explained by differences in construction of ionization sources and different chromatographic conditions such as flow rate and composition of mobile phases. Also, APPI was used for the determination of some steroid hormones such as progesterone, testosterone, androstendione, DHEA, aldesterone, and cortisol, and resulted in higher signal intensity compared with APCI and ESI [24, 25]. In our study, these ionization techniques were tested by analysis of standard solution and fortified liver samples under the same chromatographic conditions described above. As a result, ESI provided higher signal sensitivity for all analytes.

Methanol and water as mobile phases and reversed phase chromatography column (Agilent Pursuit 3 C18) were chosen as one of the most frequently used in previous works [9]. Addition of formic acid (0.1 %, v) did not increase the signal intensity in positive ionization mode. The optimized gradient program in total time of 40 min for positive and 23 min for negative modes allowed separating of isobaric analytes and matrix constituents from the hormone molecules.

Validation

From the data obtained during the validation experiment, the combined uncertainties (u_c) were calculated in accordance with Eurolab guideline [26] using Equation 1:

$$u_c = \sqrt{\frac{s_r^2}{m} + \frac{S_{wR}^2}{n} + u_{ref}^2 + \Delta^2} \quad (1)$$

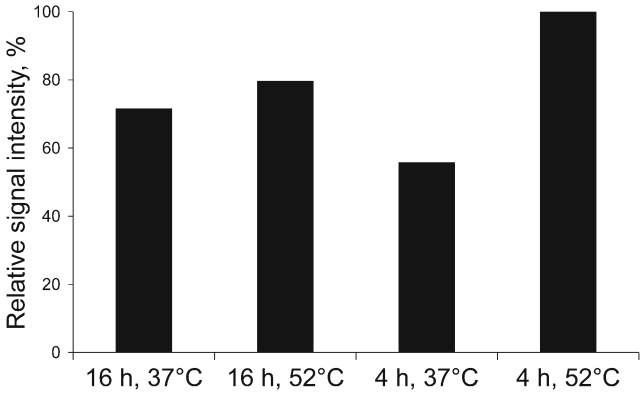

Fig. 2 The relative signal intensity of analytes (%) for different times and temperatures of enzymatic hydrolysis of α-trenbolone conjugates

where s_r is the repeatability standard deviation; S_{wR} is the within-laboratory reproducibility standard deviation; u_{ref} is the the uncertainty of analyte concentration in fortified sample (uncertainty of the reference value); Δ is the mean deviation from the reference value (bias), m is the number of replicates; n is the number of experiments (runs).

The combined measurement uncertainty corresponds to the standard deviation of the within-laboratory reproducibility plus the uncertainty of the recovery correction and comprises all single measurement uncertainty components like the uncertainty of the run, the repeatability, and the reference value. The uncertainty of the reference value was calculated following EURACHEM/CITAC approach [27] and was taken into account at a constant value of 2 %. It is comprised of the uncertainty contribution of the purity of the standard, the standard weight, and the volume of the solution. The values of combined uncertainty and recovery are shown in Table 4.

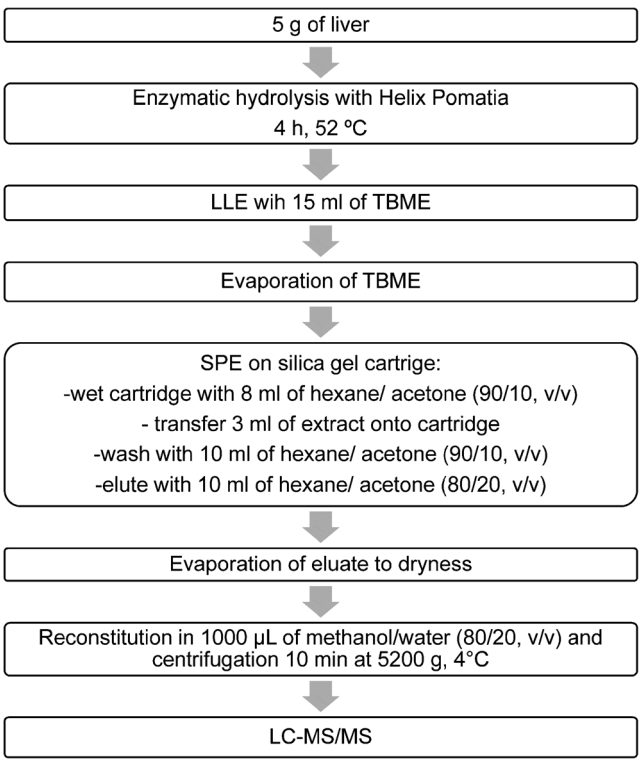

Fig. 3 Sample preparation

The limits of quantification (showing signal to noise ratio of 10) for α-trenbolone, β-trenbolone, zeranol, and taleranol were 0.5 µg kg^{-1}, and for α-nortestosterone, β-nortestosterone were 2 µg kg^{-1}.

The specificity of the method was demonstrated as no interfering peaks were observed at the retention time of analytes in a variety of blanks (Figure 4). Method was found to be linear for all analytes in the investigated range with a regression coefficient (r) at least 0.99. The recovery, corrected by matrix-matched calibration and the use of internal standards, lies in the range of 85 %–118 % for all analytes. The combined uncertainty lies below 25 % for all validation levels.

The ruggedness of method was investigated and proven by application of the different factor combinations. The method proved to be robust with regard to the two factors: operator and storage time for extracts after sample preparation.

To evaluate the effect of suppression or enhancement of analyte response attributable to co-eluting matrix components during sample preparation, the matrix effect was calculated. It

Table 4 Validation parameters for liver samples

Compound	Validation level, µg kg^{-1}	Recovery, %	u_c, %
α-Trenbolone	0.5	118	16
	2	113	13
	8	110	10
	30	112	13
β-Trenbolone	0.5	96	8
	2	103	6
	8	101	4
	30	108	8
α-Nortestosterone	2	85	21
	8	93	15
	30	92	23
β-Nortestosterone	2	104	7
	8	100	4
	30	105	5
Zeranol	0.5	117	18
	2	106	7
	8	106	9
	30	98	4
Taleranol	0.5	109	9
	2	112	11
	8	110	10
	30	110	10

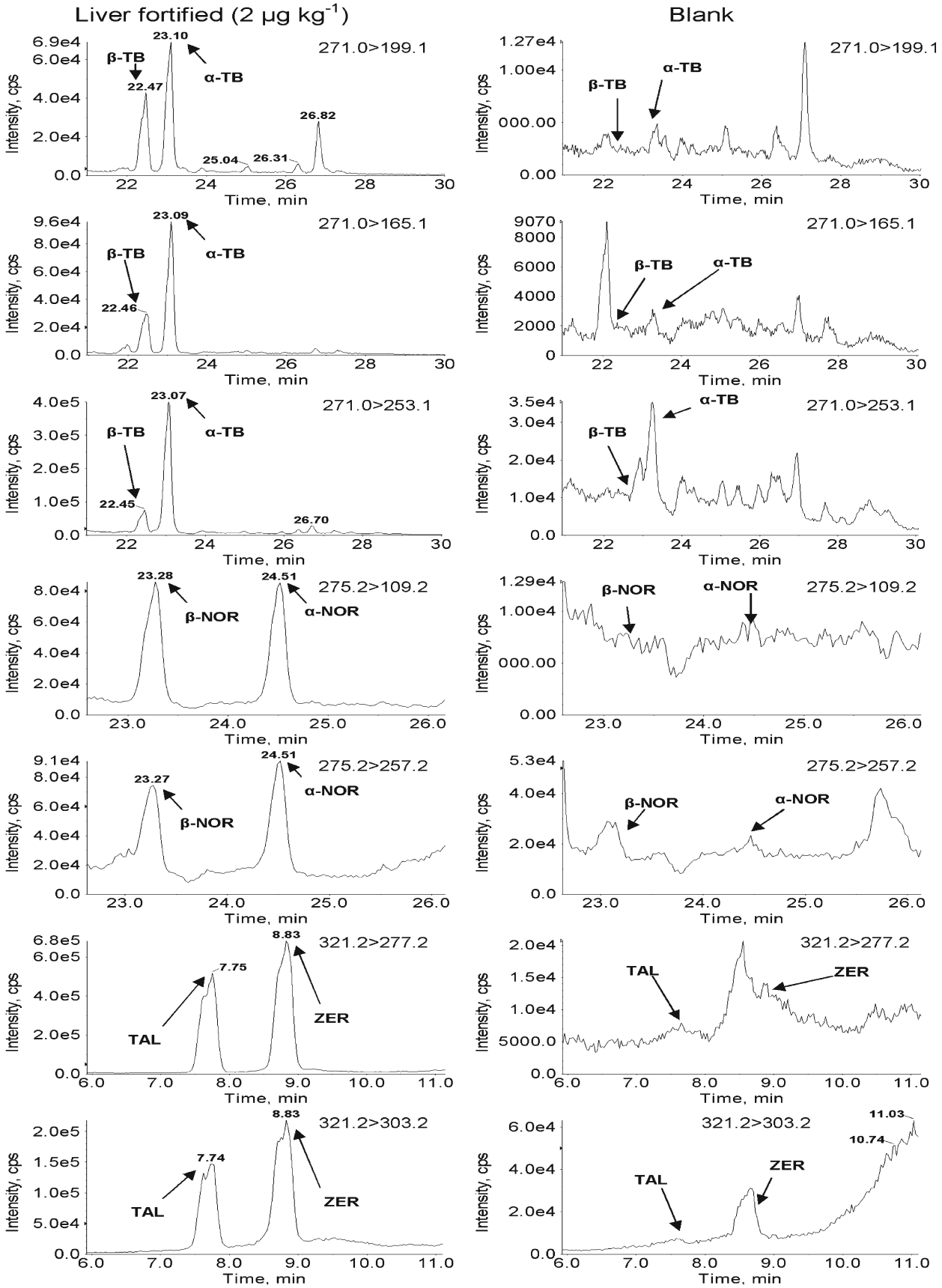

Fig. 4 Selected reaction monitoring ion chromatogramms obtained from a liver fortified at 2 µg kg^{-1} (left) and a liver blank (right)

was negligible for all analytes except for α-nortestosterone (19 %) resulting in lower accuracy compared with other analytes (Table 4).

Application to other matrices

The developed method with changes concerning the sample size and hydrolysis was also validated for meat and serum samples using experimental design described above. The sample sizes were 10 g and 5 mL for meat and serum, respectively. The hydrolysis step was omitted. The limits of quantification (showing signal to noise ratio of 10) for α-trenbolone and β-trenbolone were 0.05 μg kg^{-1} in meat, 0.1 μg L^{-1} in serum; for zeranol, taleranol, α-nortestosterone, and β-nortestosterone, they were 0.2 μg kg^{-1} in meat, 0.1 μg L^{-1} in serum. The calculated regression coefficients (r) of matrix-matched calibration curves were greater than 0.990. The specificity of the method was demonstrated by the analysis of different blanks of meat and serum samples. The recovery, corrected by matrix-matched calibration and the use of internal standards, lies in the range of 95 %–122 % for all analytes. The combined uncertainty lies below 20 % for all validation levels (Tables 5 and 6).

For urine, the developed method was applied to the analysis of α-trenbolone in FAPAS proficiency test 02220 with z-score 0.3.

Table 5 Validation parameters for meat samples

Compound	Validation level, μg kg^{-1}	Recovery, %	u_c, %
α-Trenbolone	0.05	100	9
	0.2	107	8
	1	103	7
	5	108	8
β-Trenbolone	0.05	113	15
	0.2	109	10
	1	105	6
	5	108	8
α-Nortestosterone	0.2	108	11
	1	110	10
	5	109	9
β-Nortestosterone	0.2	109	9
	1	108	9
	5	106	6
Zeranol	0.2	122	18
	1	113	12
	5	103	4
Taleranol	0.2	105	6
	1	104	5
	5	103	4

Table 6 Validation parameters for serum samples

Compound	Validation level, μg kg^{-1}	Recovery, %	u_c, %
α-Trenbolone	0.1	101	6
	0.5	98	5
	2	104	5
	8	101	3
	30	104	5
β-Trenbolone	0.1	108	10
	0.5	106	6
	2	111	11
	8	105	5
	30	107	7
α-Nortestosterone	0.1	106	8
	0.5	114	14
	2	115	14
	8	107	10
	30	100	8
β-Nortestosterone	0.1	115	15
	0.5	117	15
	2	121	20
	8	117	15
	30	102	4
Zeranol	0.1	119	17
	0.5	117	16
	2	117	16
	8	108	9
	30	95	8
Taleranol	0.1	111	12
	0.5	113	13
	2	115	14
	8	109	10
	30	104	5

Conclusions

A rapid LC-ESI-MS/MS method for the determination of α-trenbolone, β-trenbolone, α-nortestosterone, β-nortestosterone, zeranol, and taleranol in bovine liver has been developed and validated. The sample preparation of the developed method includes a 4 h enzymatic hydrolysis step, liquid–liquid extraction with MTBE and SPE on silica gel cartridge, thus making it more suitable for routine analysis in comparison with previously published methods [10–15]. The method validated in the range of 0.5–30 μg kg^{-1} for α-trenbolone, β-trenbolone, zeranol, and taleranol; and 2–30 μg kg^{-1} for α-nortestosterone and β-nortestosterone. Combined uncertainty of measurements was in the range of 4 %–23 %. The percentage of ion suppression caused by matrix was in the range of 1 %–19 %. The method proved to be robust with regard to the two factors: operator and storage time for extracts after sample

preparation. With some changes, method applied to analysis of meat and serum samples resulted in higher accuracy and sensitivity compared with liver samples.

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